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Comparative proteomic analysis of virulent and rifampicin-attenuated *Flavobacterium psychrophilum*

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Abstract

Flavobacterium psychrophilum is the aetiologic agent of bacterial coldwater disease and rainbow trout fry syndrome. In this study, we compared a wild-type strain (CSF 259-93) with a rifampicin-resistant strain and virulence-attenuated strain of *F. psychrophilum* (CSF 259-93B.17). The attenuated strain harboured a mutation in the *rpoB* gene consistent with resistance to rifampicin. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry demonstrated an altered proteome with eight proteins characteristic for the parent strain and six that were unique to the attenuated strain. Immunoblotting with a diagnostic monoclonal antibody (FL-43) identified a putative antigen (FP1493) that was subsequently cloned, expressed as a recombinant protein and confirmed as recognized by FL-43. 2D-PAGE, immunoblotting with rainbow trout, *Oncorhynchus mykiss* (Walbaum), convalescent antisera and mass spectrometry of bacterial whole-cell lysates revealed several uniquely expressed immunoreactive proteins including FP1493. An FP1493 recombinant subunit vaccine was tested, but did not provide protection against challenge with the CSF259-93 strain. While the exact mechanism responsible for altered protein synthesis and attenuation of CSF 259-93B.17 is still unknown, the differentially expressed immunoreactive proteins are a valuable resource to develop subunit vaccines and to identify proteins that are potentially involved in disease.

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Introduction

Flavobacterium psychrophilum is a Gram-negative bacterium and the causative agent of bacterial coldwater disease (CWD) and rainbow trout fry syndrome (RTFS). This pathogen is a significant concern in salmonid aquaculture because of its global distribution, high mortality rates, ability to transmit vertically and horizontally and its potential to acquire antibiotic resistance (Holt 1987; Holt, Rohovec & Fryer 1993; Michel, Antonio & Hedrick 1999). Consequently, there is significant demand for an effective vaccine that can be efficiently administered in large-scale aquaculture settings. There are, however, no viable commercial vaccines available at this time. Nevertheless, in the past few years, there have been numerous experiments focused on the identification and testing of specific proteins or protein fractions that might be used for vaccine development (Rahman *et al.* 2002; Merle *et al.* 2003; LaFrentz *et al.* 2004, 2007; Massias *et al.* 2004; Crump *et al.* 2005, 2007; Dumetz *et al.* 2007, 2008; Sudheesh *et al.* 2007; Plant, LaPatra & Cain 2009; Plant *et al.* 2011). Some of these vaccine candidates have induced protection against challenge with

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F. psychrophilum (Rahman *et al.* 2002; LaFrentz *et al.* 2004; Dumetz *et al.* 2006; Crump *et al.* 2007), but their reliance on adjuvants or delivery by injection make these vaccines costly and troublesome for the mass immunization of fish.

Sequencing of the *F. psychrophilum* genome, improvements in culturing techniques and the application of two-dimensional polyacrylamide gel electrophoresis (PAGE) have highlighted possible proteins involved in the virulence of this important fish pathogen (Duchaud *et al.* 2007; Dumetz *et al.* 2008; LaFrentz *et al.* 2009). Additionally, the recent development of a live-attenuated strain of *F. psychrophilum* (CSF 259-93B.17) generated by passage in the presence of rifampicin (LaFrentz *et al.* 2008) poses a unique opportunity to identify proteins that are potentially important for the virulence of *F. psychrophilum* and/or important for eliciting protective immune responses by the host. When grown *in vitro* under identical conditions, the parent (CSF 259-93) and rifampicin-attenuated *F. psychrophilum* (CSF 259-93B.17) strains exhibit similar carbohydrate profiles, but sodium dodecyl sulphate (SDS)–PAGE analysis of whole-cell lysates indicates differences in the profile of synthesized proteins (LaFrentz *et al.* 2008). The aim of this study was to compare protein synthesis profiles from the parent and attenuated strains of *F. psychrophilum* and to identify proteins that are immunoreactive with convalescent sera from rainbow trout, *Oncorhynchus mykiss* (Walbaum).

Materials and methods

Bacterial culture

Previously frozen *F. psychrophilum* strains CSF 259-93 (referred to as the parent strain) and CSF 259-93B.17 (referred to as the attenuated strain) were used for the study and were cultured for 72 h at 16 °C in 5 mL of tryptone yeast extract salts (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% $MgSO_4 \times 7H_2O$, 0.05% $CaCl_2 \times 2H_2O$, pH 7.2) broth (Holt *et al.* 1993). Bacteria were harvested by centrifugation at 8000 ***g*** for 5 min and the supernatant was removed. Bacterial pellets were then resuspended in 0.5 mL of sterile phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \times 7\ H_2O$, 1.4 mM KH_2PO_4, pH 7.2). *Escherichia coli* S17 carrying plasmid pMMB207-*fp1493* was grown at 37 °C in Luria-Bertani medium (LB; tryptone 10 g, yeast extract 5 g, NaCl 10 g in 1L of ddH_2O, pH 7.0) supplemented with 34 μg mL^{-1} of chloramphenicol (Cm). *Vibrio parahaemolyticus* NY-4 strain (Shah *et al.* 2008) with pMMB207-*fp1493* was grown at 37 °C in LB medium supplemented with 1.5% NaCl, 34 μg mL^{-1} of Cm and 100 μg mL^{-1} of ampicillin (Amp). For *in vivo* bacterial challenges, the parent *F. psychrophilum* strain was cultured in TYES broth or on TYES plates containing 1.5% agar at 15 °C for 72 h. Broth cultures were shaken at 100 rpm, and bacteria were harvested by centrifugation at 4 °C for 15 min at 4300 ***g***. The pellet was resuspended in PBS, and the optical density (OD) set at either 0.2 or 0.4 at 600 nm for challenge trials.

One-dimensional SDS–PAGE

Protein electrophoresis followed Laemmli (1970) with some modifications. Prior to the electrophoresis, PBS suspensions of parent and attenuated strains (OD600 of 1.5) were diluted 1:2 in sample buffer containing a reducing agent (100 mM β-mercaptoethanol) and boiled for 5 min. Proteins from whole-cell lysates or *F. psychrophilum* culture supernatants were separated using precast 4–20% polyacrylamide gradient gels (Bio-Rad). Gels were used in a Mini-PROTEAN 3 electrophoresis cell (Bio-Rad) at 120 V for 70 min. Proteins were stained with Coomassie blue, and Precision Plus protein standards (Bio-Rad) were used to estimate the molecular mass of proteins.

Two-dimensional PAGE

Whole-cell lysates of each *F. psychrophilum* strain were prepared by adding 100 μL of Nonidet P40 lysing buffer [0.5 M Tris pH 8.8, 50 mM EDTA, 10 mM nonylphenol poly(ethylene glycol ether)$_n$ (NP-40) and protease inhibitors (0.1 mM phenylmethylsulphonyl fluoride, PMSF and 100 μM N-alpha-tosyl-L-lysine chloromethyl ketone, TLCK) in PBS pH 7.4] followed by mixing, 5-min incubation on ice and sonication in a cuphorn sonicator (energy of 200 W, 30 s bursts). Proteins were extracted with alkylation reduction with tributyl phosphate (TBP) and iodoacetamide (Bio-Rad) according to the manufacturer's instructions. Precipitated proteins were washed, dried and resuspended in 100 μL of rehydration buffer [8 M urea, 50 mM dithiothreitol (DTT), 4% CHAPS and 0.25% Bio-Lyte 3/10 ampholytes]. Protein

concentration was estimated by using a QuickStart Bradford Kit (Bio-Rad). Samples from each strain with ~ 250 μg μL^{-1} protein per strip were applied to immobilized pH gradient (IPG) strips (11 cm, pH 3–10) by passive rehydration for 1 h. Rehydrated strips were covered with mineral oil and incubated overnight at room temperature. First dimension isoelectric focusing (IEF) was conducted using a Protean IEF Cell (Bio-Rad) with 0–250 V for 15 min, 0–8000 V rapid ramp for 45000 Vh and 50 μA per strip. IPG strips were subsequently treated with DTT (2% w/v) and iodoacetamide (2.5% w/v) equilibration buffers (6 M urea, 0.357 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol) with gentle rocking for 10 min. Second dimension separation was accomplished by the application of IPG strips onto polyacrylamide gels with a linear 4–20% Tris-HCl gradient and electrophoresis with a Criterion Gel System (Bio-Rad). Gels were stained with SyPro Ruby Protein Stain according to the manufacturer's directions. Digital images were collected using a Fluor-S Multi Imager (Bio-Rad), and 2D-PAGE qualitative protein patterns were compared between the strains with Quantity One software (Bio-Rad). We focused our attention on protein spots that were unique to either the parent or attenuated strain. Proteins of interest were excised and identified by mass spectrometry (MS) analysis (see below).

Western blot

Transfer of proteins from SDS–PAGE and 2D-PAGE gels to PVDF membranes (Immobilon-P, Millipore) was conducted with the Wet/Tank Criterion Blotting System (Bio-Rad) in Towbin's electrotransfer buffer (0.025 M Tris, 0.192 M glycine, 20% methanol in ddH_2O) at constant 200 mA for 90 min with cooling. Transfer blots were dried overnight and covered between Whatman blot papers. The next day, proteins on blots were stained with SyPro Ruby Blot (Bio-Rad) according to the manufacturer's recommendations and protein spots were visualized with a Fluor-S Multi Imager. Protein blots were then blocked with 5% non-fat dry milk in PBS pH 7.4 with 0.05% Tween-20 (PBS-T, pH 7.4) for 90 min and then incubated in PBS-T at room temperature for 60 min with a 1:1000 dilution of sera from production rainbow trout that had survived a CWD epizootic. Next, blots were washed 3 × 5 min with PBS-T and incubated at room temperature with 1:5000 dilution WARR mAb 1.14 (DeLuca, Wilson & Warr 1983) for 60 min with gentle shaking and washed 3 × 5 min with PBS-T. Finally, blots were incubated at room temperature for 60 min (gentle shaking) with anti-mouse IgG HRP-conjugate (1:10 000 dilution; Sigma), washed 3 × 5 min with PBS-T and visualized with a TMB Substrate Kit (Thermo Fisher Scientific). Detection of FP1493 in bacterial culture and supernatants was performed with anti-His-tag mAB (Sigma) and with FL-43 mAb (Lindstrom *et al.* 2009). Western C protein standards were detected with Strep-Tactin HRP mAb (Bio-Rad). All antibodies used for immunoblotting were diluted into blocking buffer containing 5% non-fat dry milk in PBS pH 7.4.

Protein extraction and LC-MS/MS analysis

Individual protein spots from 2D-PAGE analysis of the parent and attenuated strains that were immunoreactive with sera from convalescent fish were excised from SyPro Ruby stained gels using sterile scalpels followed by in-gel trypsin digestion. Briefly, each excised gel spot was washed in 50 μL of 100 mM ammonium bicarbonate and dehydrated in 100% acetonitrile (ACN). The gels were rehydrated with 50 μL of 10 mM DTT in 100 mM ammonium bicarbonate. Cysteine residues were reduced by heating at 56 °C for 30 min and alkylated by incubating the samples with 55 mM iodoacetamide in 100 mM ammonium bicarbonate. Samples were washed in 100 mM ammonium bicarbonate, treated with ACN once again and dried under vacuum. The samples were rehydrated in digestion buffer containing 50 mM ammonium bicarbonate and sequencing grade modified trypsin and incubated overnight at 37 °C. Next, samples were extracted with 60% ACN/1% trifluoroacetic acid (TFA) for 30 min and sonicated for 10 min. The extractions and sonications were then repeated with 3% TFA. LC-MS/MS of the peptide digests were performed on an Esquire HTC electrospray ion trap (Bruker Daltonics) and LC Packings Ultimate Nano high-performance liquid chromatography system with LC Packings monolithic column (Washington State University Molecular Biology Core).

rpoB sequencing

Genomic DNA was isolated from 5 mL cultures of the parent and attenuated strains of *F. psychrophilum*

with a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. External primers for *rpoB* amplification were 5′-AAAA TCGGAACGGATTACGG-3′ and 5′-TTTTGAA TTGTTTTTAAAGAGGTATTG-3′, and PCR involved 35 cycles of 94 °C for 30 s, 45 °C for 30 s and 68 °C for 4 min. Primers used to amplify internal *rpoB* segments for sequencing are listed in Table 1. All reactions included 2 mM $MgSO_4$, 1× PCR buffer, 0.2 mM dNTP mixture, 1 U *Taq* polymerase (Invitrogen) with 5 ng DNA template and 0.1 μM of each primer in a 50 μL reaction volume. Sequence traces were processed using Sequencher 4.10.1 (Gene Codes Corp.).

Sequencing and evaluation

Sequence analysis was performed using the BLAST and PSI-BLAST algorithms (Altschul *et al.* 1997). LC-MS/MS peptide sequences were compared using a local Mascot server (http://www.matrixscience.com) and searched against Swiss-Prot, UniProt, NCBI-GenBank and other public databases. Significant hits were assigned probability-based scores based on ion scores for peptides. Individual ion scores > 24 ($P < 0.05$) indicate identity or extensive similarity. Subsequent protein scores were derived from ion scores as a non-probabilistic basis for ranking protein hits. Identifications were considered positive if at least two peptides per protein were identified with a significant Mascot score ($P < 0.05$). Peptide sequences were also compared to a *F. psychrophilum* JIP02/86 (ATCC 49511) (GenBank # AM398681) and a CSF 259-93 draft genome sequence data (G. Weins, unpublished data, ERGO Integrated Genomics). Theoretical molecular weights and isoelectric points were estimated with Compute pI/MW algorithm (ExPASy Proteomic Server).

Table 1 Primers used for the sequencing of *rpoB* from *Flavobacterium psychrophilum*

Name	Sequence
SEQ R1	5′-TTCCTTGAAGACGGAACACC-3′
SEQ F1	5′-CTCCGTCGAAAATTGGTGTT-3′
SEQ R2	5′-AAACGGAAAAACATCGCAAG-3′
SEQ F2	5′-GCAACCCATTGTCCTCTTGT-3′
SEQ R3	5′-TGATTAGAATTGGTGCCGAAG-3′
SEQ F3	5′-AAGATGGCACGAAGCAATTT-3′
SEQ R4	5′-GATAGAACGGATGCCGAAAG-3′
SEQ F4	5′-TCCTTCACAAAGCACTTGTCC-3′
SEQ R5	5′-TGCCGAAGAAGAAGAAGGAA-3′
SEQ F5	5′-AGGCGCAACATCGGTATAAT-3′
SEQ R6	5′-CGTTGGTTTGGCTCGTATG-3′
SEQ F6	5′-TCTCGGCTAATGGATTGGTT-3′
SEQ R7	5′-CGTCGATGAAGATACTGGTGAA-3′
SEQ F7	5′-TGTGCAACAAGATAGATTTCACG-3′
SEQ R8	5′-GTGTGCCACTAAAAGCACGTT-3′
SEQ F8	5′-CCATTTGCATGGAATGATTG-3′

Expression of recombinant FP1493

The gene encoding Fp1493 (NCBI-GeneID: 5300029) was amplified from genomic DNA extracted from the parent strain (Qiaprep Spin Miniprep Kit; Qiagen) by PCR. Primer sequences used for amplification were forward: 5′-AGAGC TGCAGTTAATGGTGATGGTGATGGTGAAA TGATGTCGTGCTATTAGCCT-3′ and reverse: 5′-AGAGGGATCCCGCTGGAAAGGCATTGT ATT-3′. The amplicons were cloned into pMMB207 (Cm^R) and transformed into *E. coli* S17. Successful transformants were confirmed by PCR and restriction digestion (BamH I with Pst I) and used for conjugation with *Vibrio parahaemolyticus* NY-4 (Shah *et al.* 2008). Confirmed transconjugates were used for the purification of FP1493 protein by metal–ion affinity chromatography on columns (Bio-Rad) with cobalt resin following the manufacturer's recommendations (Thermo Scientific). Fractions of purified protein were dialysed overnight against PBS pH 7.4 and concentrated with Amicon Ultra-10 centrifugal filter units (Millipore-Amicon). Purity of FP1493 was assessed using SDS–PAGE and immunoblotting with anti-His (Sigma) and FL-43 mAbs. Protein concentrations were estimated with QuickStart Bradford (Bio-Rad) and Micro BCA Protein Assay (Thermo Scientific) kits.

Fish and rearing conditions

Rainbow trout (mean weight 2 g; outbred stock) were used for immunization trials. Fish were supplied with flow through, spring water treated with ultraviolet light at 15 °C. Fish were maintained in 378-L tanks and fed pelleted trout food (Clear Springs Foods) at 1% body weight per day. The fish stock used in this study had no previous history of exposure to *F. psychrophilum*.

Recombinant protein immunization

Purified recombinant FP1493 (rFP1493) or PBS only were mixed with equal amounts of Freund's Complete Adjuvant (FCA; Sigma) and emulsified

by vortexing. Prior to immunization 10 rainbow trout were anaesthetized by immersion in 50 mg L^{-1} tricaine methane sulphonate (MS-222; Argent). Groups of 150 fish were i.p. injected (30 gauge needle) with 50 μL of PBS/FCA or rFP1493/FCA. Fish received a total of 25 μg of protein in 25 μL PBS to give a total of 50 μL when combined with FCA. Blood before immunization was collected by severing the caudal peduncle and collection into heparinized haematocrit tubes (Chase Scientific Glass), and pooled into separate five fish pools. Serum was harvested after centrifugation at 15000 ***g*** for 5 min at room temperature and stored at −80 °C. Four weeks post-initial immunization, the fish were booster immunized with either PBS/FCA or rFP1493/FCA identically to the initial immunization as described above. This study was carried out twice using identical methods.

Bacterial challenge with CSF 259-93

At 8 weeks post-initial immunization, fish were challenged with 25 μL of *F. psychrophilum* by subcutaneous injection with a 30 gauge needle posterior to the dorsal fin, at the dorsal midline. Duplicate groups of 25 fish from each treatment were challenged with *F. psychrophilum* at an OD600 of 0.2 or 0.4, or PBS as a mock infected control. An OD600 of 0.2 correlates to 2×10^8 colony-forming units (cfu) mL^{-1} and an OD600 of 0.4 is equivalent to 5×10^8cfu mL^{-1} (Holt 1987). Mortalities were recorded daily for 28 days and spleen tissue streaked onto TYES agar to confirm the presence of yellow-pigmented bacteria. Prior to challenge, 15 rainbow trout from each group were bled and blood was pooled into five pools from three fish each. Pooled serum was used to detect antibodies to *F. psychrophilum* whole-cell lysate and rFP1493 by ELISA. The challenge and sampling protocols were identical for both studies.

Enzyme-linked immunosorbent assay (ELISA)

Serum from fish in the recombinant protein study was used in an ELISA to detect rFP1493 and *F. psychrophilum* proteins. Purified rFP1493 or *F. psychrophilum* whole-cell lysate was used as coating antigen. To produce the whole-cell lysate, the parent strain of *F. psychrophilum* was cultured in TYES broth for 96 h at 16 °C and the suspension harvested by continuous centrifugation at 8000 ***g***. The resultant bacterial pellet was resuspended in PBS, aliquoted, and alternately frozen and thawed 10× in liquid nitrogen and in a 37 °C water bath. Long-term storage of the lysate was at −80 °C. The ELISA was performed as previously described (LaFrentz *et al.* 2002). Plates were coated with 10 μg mL^{-1} of antigen in 100 μL of carbonate coating buffer, whether pure protein or whole-cell lysate. The ELISA titre was defined as the reciprocal of the dilution with an OD at least two times the negative PBS control.

Statistical analyses

The mean cumulative per cent mortality (CPM) and antibody titres from all challenges were analysed by Student's *t*-test. Differences were considered statistically significant when $P < 0.05$. Data were analysed using Minitab software [Minitab 16 Statistical Software (2010), State College, PA: Minitab, Inc. (http://www.minitab.com)].

Results

rpoB sequencing

Sequence analysis of *rpoB* from the parent and attenuated *F. psychrophilum* strains with our set of primers (Table 1) identified a single point mutation (T > C) resulting in a single amino acid substitution of glutamine (Q) residue 473 to arginine (R).

Characterization of protein profiles

Initial analysis of the rifampicin-attenuated CSF259-93B.17 strain conducted by LaFrentz *et al.* (2008) indicated that this strain exhibits an altered protein expression profile as compared to the parental CSF 259-93 strain. Our 2D-PAGE comparative analysis of whole-cell lysates prepared from the parent and attenuated strains grown under *in vitro* conditions confirmed these differences. There were a number of proteins synthesized differently between the two strains with eight unique proteins detected from the parent CSF 259-93 strain and six detected from the rifampicin-attenuated CSF 259-93B.17 strain that was also reactive with rainbow trout convalescent sera (data not shown). Proteomic analysis (Table 2) demonstrated that the attenuated strain exhibited increased synthesis of highly immunogenic OmpA P60 and Hsp70 proteins that have already been tested as possible subunit candidates (Dumetz *et al.* 2007; Plant *et al.* 2009). Other

Table 2 Proteins differentially expressed in the parent and attenuated *Flavobacterium psychrophilum* strains as identified by LC-MS/MS. Spot numbers refer to proteins in Figs 1 & 2; pI – isoelectric point

Spot number[a]	Putative protein identity	NCBI access number[b]	Predicted mass (kDa) per pI	Peptides matched (sequence coverage) (%)	Mascot score[c]
1_{WT}	EF-Tu	FP1184	43.2/5.14	6 (29)	1803
2_{WT}	FspA	FP2019	21.3/9.35	4 (38)	188
3_{WT}	Omp 121 family outer-membrane protein	FP1199	115/8.94	7 (15)	91
4_{WT}	Protein of unknown function	FP1493	22.7/8.61	5 (50)	1860
5_{WT}	RpsA	FP1793	75.7/5.72	6 (17)	183
6_{WT}	Protein of unknown function	FP1496	36.3/8.77	7 (35)	156
7_{WT}	PpiC	FP1908	33.5/6.02	6 (38)	100
8_{WT}	YueD	FP1165	27.8/7.68	4 (16)	64
9_{B17}	OmpA (P60)	FP0156	49.9/4.87	5 (16)	108
10_{B17}	DnaK/Hsp70	FP0864	67.3/4.83	13 (32)	626
11_{B17}	RpsB	FP0454	30.9/8.92	8 (35)	392
12_{B17}	FusA	FP1341	79.4/5.12	15 (56)	122
13_{B17}	Protein of unknown function	FP0261	34.8/8.46	4 (21)	526
14_{B17}	Acyl-CoA dehydrogenase family protein	FP1726	66.2/5.17	6 (13)	50

[a]Subscripts of spot numbers indicate: WT = CSF 259-93 and B17 = CSF 259-93B.17 strains.
[b]Accession number for *F. psychrophilum* genome sequence.
[c]A higher Mascot score represents greater confidence in the predicted protein match.

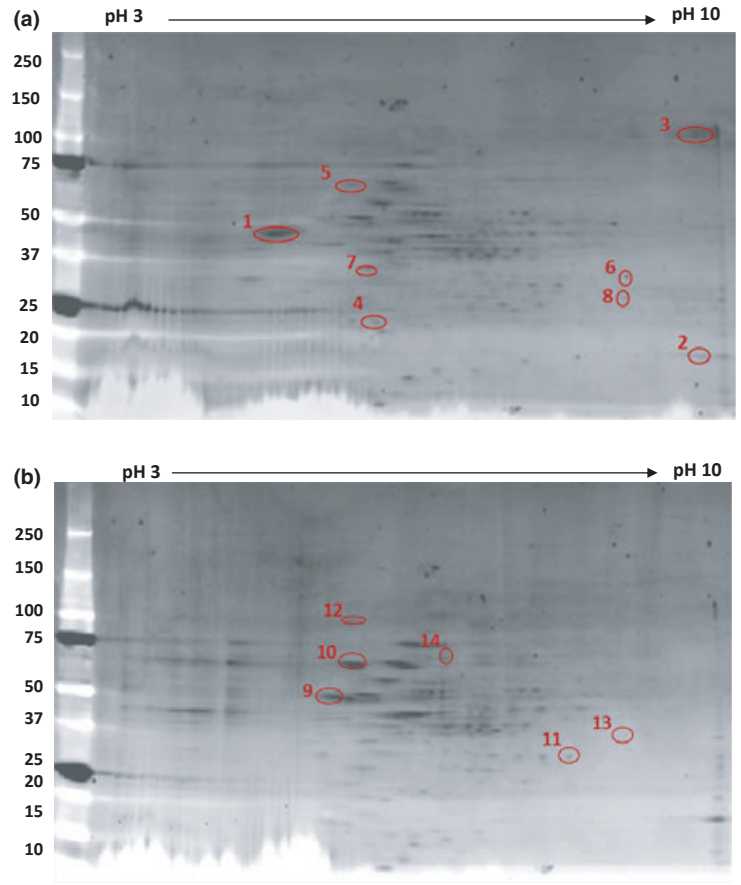

Figure 1 Analysis of whole-cell lysate of *Flavobacterium psychrophilum* strains CSF 259-93 (a) and CSF 259-93B.17 (b) by 2D-polyacrylamide gel electrophoresis. Proteins were visualized with SyPro Ruby stain, and protein spots that are differentially expressed between these two strains are circled and their numbers correspond to proteins identified by LC-MS/MS (Table 1). Molecular mass markers (kDa) are indicated on the left.

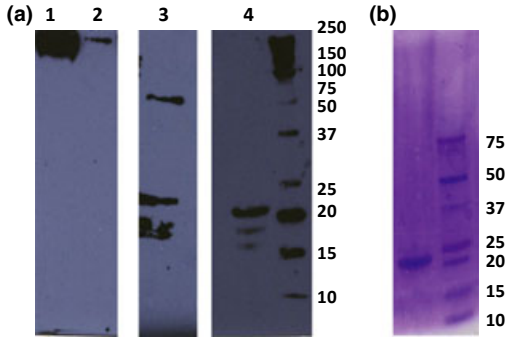

Figure 2 (a) Western blots probed with FL-43 mAb (lane 1 = CSF 259-93 lysate; lane 2 = CSF 259-93 culture supernatant; lane 3 = multimerizing recombinant FP1493); lane 4 = recombinant FP1493 probed with anti-His-tag mAb; lane 5 = molecular mass marker (kDa). (b) Sodium dodecyl sulphate–polyacrylamide gel electrophoresis analysis of purified rFP1493 (22.7 kDa).

proteins synthesized primarily by the rifampicin-attenuated strain included proteins engaged in protein expression [30S ribosomal protein S2 (*rpsB*), elongation factor G (EF-G, *fusA*)], metabolism – FP1726 (Acyl-CoA dehydrogenase family protein) and a protein with unknown function (FP0261). Another difference between the two strains came from 2D-PAGE immunoblotting with FL-43, a monoclonal antibody used for CWD diagnostics (Lindstrom *et al.* 2009). Because of the immunoreactivity of FP1493 with convalescent sera and with FL-43, and its observed ability to form high molecular mass multimers (Fig. 2), a feature possibly important for protection (LaFrentz *et al.* 2004), we selected and investigated this protein as a candidate for a subunit vaccine against CWD. The combined approach of using convalescent sera antibodies and 2D-PAGE for the selection of potential target antigens from *F. psychrophilum* was demonstrated to be useful in the past (Sudheesh *et al.* 2007). Additionally, to circumvent problems with the expression of *F. psychrophilum* proteins, we applied a non-*E. coli* protein expression system (Shah *et al.* 2008).

Immunization with rFP1493

Immunization with rFP1493 in two independent trials did not demonstrate a protective effect of the protein (Table 3). The CPM of fish injected either with PBS or rFP1493 and challenged with the virulent *F. psychrophilum* parent strain was not significantly different ($P = 0.98$ for OD600 0.2 and $P = 0.19$ for OD600 0.4 in trial 1, and in trial 2, $P > 0.05$ for both OD600 0.2 and 0.4). There were no mortalities in the mock control groups. Specific antibody responses against rFP1493 and *F. psychrophilum* whole-cell lysate were measured by ELISA. No significant differences in antibody titres against *F. psychrophilum* whole-cell lysate were observed between groups (data not shown). In both independent trials, significant differences ($P < 0.05$) in rFP1493 specific antibody titres were demonstrated between fish immunized with PBS and rFP1493 ($P < 0.007$, Table 3).

Discussion

Live vaccines, especially ones attenuated with rifampicin, have successfully conferred protection against several important bacterial diseases of fish including edwardsiellosis, enteric septicaemia of catfish and columnaris disease (Klesius & Shoemaker 1999; Shoemaker, Klesius & Evans 2002; Lawrence & Banes 2005; Sun, Liu & Sun 2010; Olivares-Fuster & Arias 2011). The live-attenuated

Table 3 Cumulative per cent mortality (CPM) ± standard error (SEM) of rainbow trout challenged with *Flavobacterium psychrophilum* CSF 259-93 strain 8 weeks post-immunization with recombinant FP1493

Treatment	Trial number	Challenge Dose OD600	Mean CPM ± SEM ($n = 2$)	Mean FP1493 specific antibody titre ± SEM ($n = 5$)
PBS + FCA	1	0.2	22.6 ± 6.6	100
		0.4	18.4 ± 2.4	
rFP1493 + FCA	1	0.2	23.3 ± 11.3	2720 ± 999*
		0.4	28.08 ± 0.8	
PBS + FCA	2	0.2	16 ± 8	< 100
		0.4	30 ± 0.8	
rFP1493 + FCA	2	0.2	16 ± 4	760 ± 240*
		0.4	22 ± 2	

FCA, Freund's Complete Adjuvant; PBS, phosphate-buffered saline.
*Indicates a significant difference from PBS + FCA $P < 0.05$.

strain CSF 259-93B.17 was also selected by rifampicin passage and it confers significant protection against challenge with CSF 259-93 (LaFrentz *et al.* 2008).

The current work identified a mutation in the *rpoB* gene consistent with resistance to rifampicin. *RpoB* encodes the β-subunit of RNA polymerase (RNAP) that is the target for the antibiotic rifampicin (Manten & Van Wijngaarden 1969; Campbell *et al.* 2001), and differential protein synthesis exhibited by the attenuated strain may correlate with the fact that CSF 259-93B.17 harbours the mutated *rpoB* gene resulting in a single amino acid substitution (Q473R) of RpoB. A similar mutation leading to a change of Q473R, which corresponds to Q513R in *rpoB* of *E. coli*, was previously described by Jin & Gross (1988).

Our comparative proteomic analysis identified 14 proteins that exhibit apparent differential synthesis consistent with the attenuated strain having an altered protein expression profile as compared to the virulent parent strain. These include the highly immunogenic outer-membrane antigen P60 (OmpA P60), heat shock protein 70 (DnaK/Hsp70) and elongation factor Tu (EF-Tu), which have been examined as possible subunit vaccines; however, these proteins did not confer significant protection to CWD (Dumetz *et al.* 2007; Plant *et al.* 2009, 2011). While Hsp70 has been documented previously from CSF 259-93 (Sudheesh *et al.* 2007), in the current study we only detected this protein for CSF 259-93B.17. This difference was reproduced consistently for several technical and biological replicates (data not shown) and may simply reflect the differences in methods that were employed herein compared with Sudheesh *et al.* (2007).

Importantly, these differentially expressed genes are not grouped in a single locus or operon, which indicates that the attenuation from rifampicin possibly resulted from a multi-factorial alteration of transcription. Analysis of an immunoreactive spot with mAb FL-43 identified FP1493, a protein of unknown function demonstrated as responsive to iron sequestration and also identified by another proteomic analysis of *F. psychrophilum* (Dumetz *et al.* 2008; LaFrentz *et al.* 2009). We were particularly interested in the FP1493 because of its apparent responsiveness to iron levels (LaFrentz *et al.* 2009) and because it appears to be associated with a HmuY and haemin-uptake gene cluster (*hmu*) homologous to *Porphyromonas gingivalis*. HmuY is a putative haem-binding lipoprotein associated with the outer membrane as a part of an operon engaged in haemin utilization and may play a significant role in biofilm accumulation (Olczak *et al.* 2008, 2010; Wojtowicz *et al.* 2009). In addition to iron-acquisition mechanisms suggested by Moller *et al.* (2005), FP1493 may also be the part of the iron-uptake system utilized by *F. psychrophilum*. If so, FP1493 may be involved in colonization, iron uptake and growth of *F. psychrophilum* under iron-limiting conditions within the host. Nevertheless, immunization with recombinant FP1493 was insufficient to protect rainbow trout from an experimental challenge with CSF 259-93 strain despite eliciting a specific antibody response against the protein as determined by ELISA. We should point out that the severity of challenge from CSF 259-93 in the current study was less than reported in previous studies (LaFrentz *et al.* 2004, 2008), but this outcome reflects the level of variance encountered with this challenge model (Plant *et al.* 2011). Importantly, even if we averaged CPM for all controls (21.8%) vs. for rFP1493 immunized fish (22.4%), there is no evidence that protection would be evident given a more severe challenge.

Members of the outer-membrane family of proteins, OmpA P60 and Omp121 could also be involved in host–pathogen interactions and play a potential role in protective immunity. OmpA P60, in particular, was intensively studied in the context of providing protection against *F. psychrophilum* (Merle *et al.* 2003; Dumetz *et al.* 2007). Additionally, a BLAST search for conserved domains reveals similarities of Omp121 with outer-protein families of membrane channels or TonB-dependent haemoglobin/transferrin/lactoferrin receptors and TonB-dependent siderophore receptors. Given its putative role in iron acquisition, it may be possible that the lack of Omp121 in the attenuated strain contributes to its inability to cause disease. The exact function of peptidyl-prolyl cis-trans isomerase (PpiC) in *F. psychrophilum* has not been established, but the protein may be a ribosome-bound trigger factor or it may be engaged in protein folding processes (Stoller *et al.* 1995; Justice *et al.* 2005; Kaiser *et al.* 2005; Maier *et al.* 2005). Flavo-specific antigen A (FspA) was demonstrated to be immunoreactive with trout convalescent sera and was tested as a possible subunit vaccine candidate against CWD (Crump *et al.* 2005). Involvement of benzil reductase (YueD), FP0216 or Acyl-CoA dehydrogenase protein FP1726 in modulating host-immune re-

sponse or virulence is unknown. Interestingly, multiple acyl-CoA synthases homologues were recently reported to be involved in virulence of *Pseudomonas aeruginosa* (Kang *et al.* 2010). EF-Tu that was identified from the parent strain and in *E. coli* has been reported to play a role in gene expression, DNA repair and protein processing (Malki *et al.* 2002). Additionally, EF-Tu was demonstrated to interact with hydrophobic regions of proteins by assisting refolding in a manner similar to molecular chaperones such as DnaK/Hsp70 (Caldas, El Yaagoubi & Richarme 1998; Malki *et al.* 2002). The parent strain exhibited increased synthesis of the 30S ribosomal subunit S1 encoded by *rpsA*, while the attenuated strain showed apparent upregulation of the 30S ribosomal subunit S2 (*rpsB*). These highly conserved proteins are involved in complex co-regulation and modulation of gene expression and protein synthesis networks (Wilson & Nierhaus 2005; Aseev *et al.* 2008). Interestingly, in *E. coli* upregulation of S2 was reported to cause suppression of the *tsf* gene, encoding elongation factor-Ts, which is a GDP/GTP exchanger for EF-Tu. With the altered function of EF-Tu, a key player in gene expression, the *E. coli* mutant overexpressing S2 revealed defective growth (Aseev *et al.* 2008). If the same is true for *F. psychrophilum*, then observed elevated levels of RpsB in the attenuated strain may be a possible cause of minor growth impairment exhibited by this attenuated strain (LaFrentz *et al.* 2008). Slight growth impairment was also the characteristic for other studies involving rifampicin-resistant strains (Moorman & Mandell 1981; Jin & Gross 1989; Mariam *et al.* 2004). Another protein with increased spot intensity found in the rifampicin-attenuated strain was the EF-G (*fusA*), which is putatively involved in gene expression and protein synthesis but can also mediate protein folding and thus exhibit chaperon-like properties (Caldas, Laalami & Richarme 2000).

Collectively, our results may shed some light not only on the virulence of *F. psychrophilum* but also indicate the possible role of RNAP in rifampicin attenuation. Rifampicin (Rif) is a broad-range antibiotic from the rifampicin group that inhibits the β-subunit of prokaryotic DNA-dependent RNAP. The drug acts by blocking the initiation of transcription (Wehrli *et al.* 1968), and rifampicin resistance is typically conferred by point mutations in *rpoB* that encodes the β-subunit. Nevertheless, the precise mechanism leading to rifampicin attenuation of bacteria is not clear, and *rpoB* mutations produce different outcomes depending on the exact mutation and the bacterial species. For example, induction of rifampicin resistance may have no effect on virulence as demonstrated by rifampicin and multi-drug resistant *Mycobacterium tuberculosis* (Ahmad & Mokaddas 2009). In the case of *Edwardsiella ictaluri* attenuated with rifampicin (RE-33), bacteria display altered LPS structure lacking a high molecular mass LPS fraction (Klesius & Shoemaker 1999). Additionally, further analysis of RE-33 revealed that genes engaged in the LPS biosynthesis pathway were not mutated, which suggests an altered protein expression programme by this strain (Arias *et al.* 2003). The attenuated strain of *F. psychrophilum* examined in this study does not exhibit different LPS or glycocalyx profiles as compared to wild-type CSF 259-93, but it apparently executes an abnormal programme of protein synthesis (LaFrentz *et al.* 2008). A phenomenon of altered protein expression was also observed in the case of rifampicin-attenuated *Edwardsiella tarda*, the aetiologic agent of septicaemic edwardsiellosis in fish (Sun *et al.* 2010) and *Flavobacterium columnare* (Olivares-Fuster & Arias 2011).

Our analysis revealed that the CSF 259-93B.17 strain harbours a mutated *rpoB* gene and executes a different protein expression pattern as compared to its parent strain *in vitro*, including synthesis of several proteins of unknown function. The potential involvement of transcriptional regulators (e.g. EF-Tu, EF-G and S2) suggests that attenuation results from an altered interaction between these regulators and the rifampicin-resistant RNAP. Our data also suggest that it is important to associate the alteration of protein synthesis with the relationship between transcriptional regulators (e.g. elongation factors and ribosomal proteins) and their interactions with normal and mutant RNAPs in *F. psychrophilum*. Identification of the exact mechanism of rifampicin attenuation will likely identify novel approaches to generate effective live-attenuated vaccines that do not rely on random mutations.

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